PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

Patent Office
Canberra

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 9752 for a patent by UNISEARCH LIMITED as filed on 24 December 2001.

COMMONWEALTH OF AUSTRALIA
PATENT OFFICE

WITNESS my hand this
Twentieth day of January 2003

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

AUSTRALIA

Patents Act 1990

Unisearch Limited

PROVISIONAL SPECIFICATION

Invention Title:

Enzymatic redox labelling of nucleic acids

The invention is described in the following statement:

Field of the invention

The present specification is directed to the synthesis, constitution and application of redox-tagged nucleoside analogues and more specifically nucleoside triphosphates for random or site-specific incorporation into nucleic acids by template-dependent nucleotidyl transferases, along with the electrochemical detection of the resulting nucleic acid products.

Background of the invention

Nucleic acids are manipulated *in vitro* in a wide variety of research and diagnostic methods. Detection of specific sequences plays a central role in the identification of genes and in analysis of their expression and variation. The methods can involve synthesis of nucleic acid probes by means of nucleotidyl transferase enzymes for the purposes of labelling or determination of base sequence identity. Labelling often involves the incorporation of a nucleotide which is chemically tagged or which is of a particular chemical composition so as to make it specifically detectable.

For many years, nucleotides and nucleic acids have been labelled with radioactive isotopes, most commonly ^{32}P. However, the use of radioactive constructs carries a potential health risk and attendant regulatory complications, with additional inconvenience caused by radiolysis, short isotope half-lives and relatively cumbersome means of detection.

In an early implementation of non-isotopic labelling, biotin-tagged nucleotides have previously been described. This application allowed efficient incorporation into DNA and RNA by the appropriate polymerases. Colourimetric detection of the label exploited the biotin-avidin interaction and an avidin-enzyme conjugate. More recently, hapten tagging methods such as digoxigenin-labelled NTPs and antibody-enzyme conjugates have been introduced as an alternative. Biotin-, digoxigenin- and dinitrophenyl-nucleotides are in now widespread use.

Currently, fluorescent tagging dominates applications in nucleic acid sequencing and microarray expression analysis. Fluorescent labelling offers increased sensitivity and the option for multicolour detection. In this as in other approaches, oligonucleotides can be labelled during chemical oligonucleotide synthesis, by incorporation of fluorescent-labelled nucleotides in the course of enzymatic synthesis or by post-synthetic derivatisation with a reactive dye construct. A broad variety of fluorescent-

tagged NTPs, dNTPs, ddNTPs and acyclo-NTPs intended for enzymatic incorporation is now commercially available. In an elaboration of fluorescent methodology, nucleotides labelled with rare earth cryptates have recently been used to implement time-resolved fluorescence and FRET detection of nucleic acids.

Electrochemical detection (ECD) is the detection of molecules on the basis of the flow of electrons. Electrochemical detection offers a promising alternative to other approaches: it can be highly sensitive, rapid and amenable to inexpensive production in miniaturized (*eg.* lab-on-chip) formats. Several different implementations are currently being developed and commercialized. In one approach, unlabelled nucleic acids are detected with amol sensitivity by transition metal complex-mediated oxidation of guanine (G) nucleobases at potentials around 1.1 V. It is likely that this approach can also be used for detection of A,C and T/U by labelling with nucleobase analogues possessing lower redox potentials than the natural species.

Most other electrochemical implementations are based upon introducing one or more copies of a redox label, typically a metal complex, metallocene or quinone, by chemical conjugation. Due to its stability, ready synthetic access and ease of redox tuning, labelling with ferrocene has been the focus of significant attention. In early demonstrations of redox tagging, 5'-aminohexyl oligonucleotides were conjugated with ferrocene to enable electrochemical detection of hybridization and PCR products at fmol levels. Similar 5'-ferrocene oligonucleotides were immobilized at their 3' ends for characterization of surface monolayers. Phosphoramidites of ferrocene for 5' terminal labelling during oligonucleotide synthesis and 3'-end labelling of oligonucleotides have also recently been demonstrated.

For internal incorporation during chemical oligonucleotide synthesis, phosphoramidite monomers with a ferrocenyl moiety linked to position 5 of 2'-deoxyuridine and on-column derivatization of iodo-dU with ferrocenyl propargylamide have been described, as have phosphoramidites labelled at the 2'-ribose position of adenosine and cytosine.

In addition to these approaches, non-specific internal labelling of DNA probes has been obtained by reaction with ferrocenecarboxaldehyde or aminoferrocene. The ability of a naphthalenediimide derivative of ferrocene to preferentially bind dsDNA via intercalation has been employed to detect hybridization.

The application of electrochemical methods to nucleic acids is not as advanced as fluorescence approaches. This field is developing with the construction of CE (capillary electrophoresis) chips with integrated ECD and the recent demonstration of a "four colour dye primer" analogue strategy for ECD DNA sequencing. However, the current art of preparing individual redox-labelled nucleic acids by phosphoramidite or post-synthesis reactions restricts the range of practical uses.

Summary of the invention

The present invention provides a modified nucleoside analogue having a redox-label at the nitrogenous base, sugar or 5'-(tri)phosphate moiety.

The present specification discusses the synthesis, constitution and application of redox-tagged nucleoside analogues and more specifically NTPs for random or site-specific incorporation into nucleic acids, along with their electrochemical detection. Importantly, these analogues can be incorporated into oligo- and poly-nucleotides by a number of polymerases (template-dependent nucleotidyl transferases) in the course of enzymatic synthesis. In some applications a high level of labelling can be achieved, allowing a significant increase in the sensitivity of detection.

Accordingly, in a first aspect the invention provides a modified nucleoside analogue having the formula (**I, II** or **III**)

I: P - S - B - L - R

II: P - S - L - R

III: R - L - P - S - B

where

P is a 5' OH, or a mono-, di, or tri-phosphate or analogue or derivative thereof,

S is a substituted or unsubstituted five- or six-membered sugar, sugar analogue or acyclo sugar analogue,

B is a substituted or unsubstituted nitrogenous base or base analogue or derivative thereof,

L is a linker group, and

R is a substituted or unsubstituted metallocene moiety or substituted or unsubstituted metal chelating agent or substituted or unsubstituted redox-active organic moiety.

An advantage of these modified nucleoside analogues is that they are capable of enzymatic incorporation into a nucleotide chain.

In one embodiment, P is OH or a phosphate-containing moiety including α–,β–, or γ– thiophosphate, phosphoramidate, dithiophosphate or other enzyme-compatible moiety.

In one preferred embodiment of the invention, group P is a triphosphate. When this is the case the nucleoside triphosphates of the present invention are most readily capable of being incorporated by enzymatic means into nucleic acid chains.

Preferably, group S is ribose, 2'-deoxyribose, 2',3'-dideoxyribose, 3'-fluoro-2'-deoxyribose or an acyclo moiety.

Preferably, group B is a substituted or unsubstituted purine or pyrimidine. More preferably, B is an adenine, guanine, cytosine, uracil, or thymine derivative including the 7-deaza variants of adenine and guanine.

Preferably, L is a saturated or unsaturated aliphatic chain, with or without cyclic groups. Preferably L is 1-24 bonds in contour length, most preferably 3-12 bonds in length.

R is a substituted or unsubstituted metallocene, a substituted or unsubstituted metal chelate or an organic redox moiety. Preferably, suitable metallocenes include ferrocene and other metallocenes with redox potentials in the range of –1.0 to +1.0 V vs. Standard Hydrogen Electrode (SHE).

In one embodiment, the redox-active organic moiety is a quinone.

Preferably, suitable metal chelates include chelates and cryptates of transition metals such as iron, copper, ruthenium and rhodium, or other non-transition elements with suitable redox behaviour.

In a preferred embodiment, R is a substituted or unsubstituted metallocene moiety, more preferably a ferrocene.

In a second aspect, the invention provides a method of synthesising a modified nucleoside analogue according to the first aspect of the invention, the method comprising reacting a nucleoside or nucleotide precursor with a metallocene, metal chelate or organic redox moiety precursor in the presence of a condensing agent so as to form a link between the nucleos(t)ide analogue and the metallocene, metal chelate or organic redox moiety.

In a preferred embodiment, the invention provides a method of synthesizing a modified nucleoside analogue according to the first aspect of the invention, the method comprising reacting a nucleoside or nucleotide precursor with a metallocene precursor in the presence of a condensing agent

so as to form a link between the nucleoside analogue and the metallocene or derivative thereof.

More preferably, the link is formed between the nitrogenous base of the nucleoside analogue and the metallocene.

In one embodiment the nucleotide precursor is 2'-deoxyuridine 5'-triphosphate.

In one embodiment the metallocene precursor is a carboxylic acid. Preferably, the metallocene precursor is ferrocenecarboxylic acid or ferroceneacetic acid or derivative thereof.

Preferably, the condensing agent is selected from any one of a carbodiimide, for example dicyclohexylcarbodiimide, uranium compounds, activated ethers and other compounds employed in the formation of amide bonds.

In a preferred embodiment the condensing agent is O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU).

In a third aspect, the present invention provides an oligo- or poly-nucleotide probe, primer or enzymatic reaction product comprising at least one residue of a metallocene nucleoside analogue according to the first aspect.

In a fourth aspect, the present invention provides a method of nucleotide chain incorporation, the method comprising reacting a template nucleotide chain with a modified nucleoside analogue according to the first aspect in the presence of a processive nucleotidyl transferase or polymerase.

In a fifth aspect, the present invention is directed to a method of nucleotide chain extension, the method comprising reacting a nucleotide chain with a modified nucleoside analogue according to the first aspect in the presence of a non-processive nucleotidyl transferase such as terminal transferase or poly(A) polymerase.

Preferably, the modified nucleoside analogue is a triphosphate.

In a sixth aspect, the present invention provides a method of electrochemical detection of DNA, RNA, DNA/RNA chimers or nucleic acid analogues, the method comprising incorporating a modified nucleoside analogue according to the first aspect of the invention into a nucleic acid chain and detecting the analogue on the basis of it's redox potential.

In an seventh aspect, the present invention provides a method of electrochemical detection of DNA, RNA, DNA/RNA chimers or nucleic acid

analogues, the method comprising incorporating two or more different modified nucleoside analogues according to the first aspect of the invention, into the same or different nucleic acid chains, and detecting the modified nucleoside analogues on the basis of their different redox potentials.

The invention will hereinafter be further described by way of the following non-limiting examples and accompanying figures.

Brief description of the drawings

Figure 1. Synthesis of ferrocene-labelled derivatives of UTP and dUTP.

Figure 2. Cyclic voltammogram of Fc-dUTP.

Figure 3. A. Structure of template-primer used for enzymatic incorporation of Fc-dUTP into DNA. B. Incorporation of Fc-dUTP into DNA by Klenow fragment and T4 DNA polymerase. The primer-template DNA of Fig. 3A was incubated with DNA polymerase and different sets of dNTPs (indicated at the top of the figure). The length of DNA fragments is shown on the left.

Figure 4: Electrochemical detection of 60 fmol Fc-dU-labelled DNA following HPLC. Lower panel: UV detection at 260 nm. Upper panel: ECD at 700 mV.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (eg., in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

In a first aspect the invention provides a modified nucleoside analogue having the formula (**I, II** or **III**)

I: P - S - B - L - R

B
|
II: P - S - L - R

III: R - L - P - S - B

where

P is a 5' OH, or a mono-, di, or tri-phosphate or analogue or derivative thereof,

S is a substituted or unsubstituted five- or six-membered sugar, sugar analogue or acyclo sugar analogue,

B is a substituted or unsubstituted nitrogenous base or base analogue or derivative thereof,

L is a linker group, and

R is a substituted or unsubstituted metallocene moiety or substituted or unsubstituted metal chelating agent or substituted or unsubstituted redox-active organic moiety.

Importantly, the modified nucleoside analogue of the present invention is capable of enzymatic incorporation into a nucleotide chain.

A nucleoside analogue is a compound which is capable of being incorporated by enzymatic or chemical means into a nucleic acid (DNA or RNA or chimeric DNA/RNA) chain, and is there capable of base stacking into the chain and base pairing or otherwise sterically accommodating a nucleotide residue in a complementary chain.

A natural nucleotide consists of a nitrogenous base, a sugar, and one or more phosphate groups. In a more general definition, a nucleotide analogue may include highly unnatural forms of these moieties, including extreme truncation of the sugar.

In the embodiment of this invention, group P is most commonly a triphosphate, or α-thio-triphosphate, but may include mono-, and di-phosphates as well as β- and γ-thiotriphosphates and other analogues such as dithiophosphates, phosphoramidates and other enzyme-compatible moieties.

In both nucleosides and nucleotides the nitrogenous base is a purine or pyrimidine derivative. The two major purines are adenine and guanine, and

the three major pyrimidines are cytosine, uracil, and thymine. The nitrogenous base may be modified. For example, for uridine the C4 substituent (O) may be replaced by S to form 4-thiouridine. For cytosine, H5 may be replaced by a methyl group to form 5-methylcytosine. In a 7-deaza purine derivative the N7 may be replaced by a C7. It is envisaged that further modifications could be made to the nucleoside derivative such that the nitrogenous base is replaced with an alternative aromatic group, for example a pyrrole or indole ring structure. Such modifications are included within the scope of the invention.

According to the present invention, the sugar structure of Formulae **I-III** may be a substituted or unsubstituted pentose or hexose or an acyclo moiety. Preferably, the pentose is a ribose, 2'-deoxyribose, 2',3'-dideoxyribose, 3'-fluororibose, 3'-aminoribose, 3'-fluoro-2'-deoxyribose, 3'-amino-2'-deoxyribose or 3'-azido-derivatives. Acyclo sugar replacements will also function as nucleotidyl transferase substrates.

R is a substituted or unsubstituted metallocene, a substituted or unsubstituted metal chelate or an organic redox moiety. In one embodiment, suitable metallocenes include ferrocene and other metallocenes with redox potentials in the range of −1.0 to +1.0 V vs. Standard Hydrogen Electrode (SHE). In an alternate embodiment, suitable metal chelates include chelates and cryptates of transition metals such as iron, copper, ruthenium and rhodium, other non-transition elements with suitable redox behaviour.

Preferably, R is unsubstituted or substituted ferrocene. Various substituents may be selected to modify the redox potential of the ferrocene nucleoside analogues thereby providing different labels. Suitable substituents include nitro groups, primary, secondary and tertiary amines, hydroxy, alkoxy, amidate, halogen, alkyl and alkyl derivatives and a range of other substituents compatible with substitution at the cyclopendadienyl ring. The redox-modifying substituents may be added to the ring of the ferrocene which is not attached to the linker group. This selectivity is caused by the electronic properties of the prior substituted ring, which directs substitution to the other ring.

Where R is a substituted metal chelate the metal ligands may also be selected to modify the redox potential of the metal chelate nucleoside analogue. This may be achieved by variation of donor atoms between oxygen, nitrogen, sulfur and other donors and by variation of ligand

framework structure. As an alternative, the metal component of a single chelate or cryptate ligand may be varied to provide a range of redox potentials

Group R is linked to the nucleoside by a linker group L. The linker group preferably a saturated or unsaturated aliphatic chain, with or without cyclic groups, preferably 1-24 bonds in contour length, most preferably 3-12 bonds in length. The degree of saturation may be varied. A higher proportion of double and/or triple bonds and/or aromatic rings gives greater rigidity. The carbon chain may be substituted with one or more nitrogen, sulphur and/or oxygen atoms. A wide range of linkage chemistries are compatible.

In a preferred embodiment, the linkage occurs via an alkyl amido group.

In a second aspect, the invention provides a method of synthesising a modified nucleoside analogue according to the first aspect of the invention, the method comprising reacting a nucleoside or nucleotide precursor with a metallocene, metal chelate or organic redox moiety precursor in the presence of a condensing agent so as to form a link between the nucleo(s/t)ide analogue and the metallocene, metal chelate or organic redox moiety.

In a preferred embodiment, the invention provides a method of synthesising a modified nucleoside analogue according to the first aspect of the invention, the method comprising reacting a nucleoside or nucleotide precursor with a metallocene precursor in the presence of a condensing agent so as to form a link between the nucleoside analogue and the metallocene.

Nucleo(s/t)ide precursors can have a variety of forms, including derivatized nucleosides and mononucleotides. The preferred reaction involves a nucleoside triphosphate and a minimum number of chemical steps A person skilled in the art can accomplish this synthesis by a number of methods.

Preferably the metallocene precursor is a metallocene carboxylic acid. In other embodiments, the metallocene precursor can also be another reactive form containing alkylamino, aldehyde, halogenated or other moieties. Preferably, when the modified nucleoside analogue is a ferrocene nucleoside analogue the metallocene precursor is ferrocenecarboxylic acid or ferroceneacetic acid.

Condensing agents are well known in the art and include dicyclohexylcarbodiimide and other carbodiimides in addition to uronium compounds, activated ethers and other compounds employed in the

formation of amide bonds. In one embodiment the condensing agent is O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU).

The nucleoside analogues of the present invention are useful for labelling DNA, RNA and DNA/RNA chimers or for incorporation into oligonucleotides.

In one aspect, the present invention is directed to an oligo- or polynucleotide probe, primer or other enzymatic reaction product comprising at least one residue of a metallocene nucleoside analogue according to the first aspect.

According to the present invention molecular probes or primers may be generated by recombinant or synthetic means. Generally the probe or primer is a polynucleotide that hybridises specifically to a target sequence. Primers include for example a PCR primer or a primer for an alternate amplification reaction.

Generally, enzymatic reaction products include any products produced by an enzymatic reaction, such as by a polymerase reaction.

In another aspect, the present invention provides a method of nucleotide chain extension, the method comprising reacting a template nucleotide chain with a modified nucleoside analogue according to the first aspect in the presence of a processive nucleotidyl transferase or polymerase.

In a further aspect, the present invention is directed to a method of nucleotide chain extension, the method comprising reacting a nucleotide chain with a modified nucleoside analogue according to the first aspect in the presence of a non-processive nucleotidyl transferase such as terminal transferase or poly(A) polymerase.

Generally, a processive nucleotidyl transferase is a transferase which uses a template to polymerase nucleotides into a complementary chain. A non-processive nucleotidyl transferase is one which is usually template-independent and which produces a nucleotide having a limited number of nucleotides.

Preferably, the modified nucleoside analogue is a nucleoside triphosphate.

The inventors present the first redox-tagged nucleoside triphosphates for labelling nucleic acids by common DNA and RNA polymerases with a

view to facilitating the preparation of electrochemically-detectable nucleic acid probes.

The ferrocene-labelled derivatives of the present invention proved to be good substrates for commonly used polymerases, thus allowing a high degree of labelling. In one embodiment the inventors have demonstrated the synthesis of derivatives modified at position C5 of the pyrimidine ring (Fig. 1) using nucleoside triphosphates. The C5 modification rarely interferes with incorporation of modified nucleotides into DNA or RNA by the majority of polymerases. Even dUTP and UTP derivatives with bulky C5 substituents can be successfully used as substrates for these enzymes. It is understood that the substrate quality of any particular nucleotide derivative will vary between polymerases.

In one aspect, the present invention provides a method of electrochemical detection of DNA, RNA, DNA/RNA chimers or nucleic acid analogues, the method comprising incorporating a modified nucleoside analogue according to the present invention into a nucleic acid chain and detecting the analogue on the basis of it's redox potential.

In another aspect, the present invention provides a method of detection of DNA, RNA, DNA/RNA chimers or nucleic acid analogues, the method comprising incorporating two or more different modified nucleoside analogues according to the present invention into the same or different nucleic acid chains, and detecting the modified nucleoside analogues on the basis of their different redox potentials. This involves the production of redox-labelled nucleotides with different redox potentials, incorporation of these nucleotides into nucleic acid, followed by simultaneous detection and quantification.

In one embodiment, labelling one type of nucleotide (eg. dUTP) with two different redox tags, followed by incorporation of these nucleotides separately into cDNAs corresponding to different treatments, mixing of the RNAs and simultaneous detection renders an electrochemical analogue of two-colour mRNA expression analysis. When redox-labelled terminator nucleotides (usually those lacking a 3'OH group, or more generally those nucleotides that cause termination of enzymatic chain elongation following their incorporation into the chain) are employed such that nucleotides corresponding to each of the four common bases A, G. C and T carry different redox groups, an electrochemical analogue of four-colour dye-terminator

nucleic acid sequencing will be enabled. In a similar embodiment, analysis of nucleic acid polymorphisms (SNPs and indels) by primer extension methods can be enabled.

A person skilled in the art of the present invention could provide the invention in a kit. The kit may contain components necessary to practice the invention. For example, a kit may contain a vial(s) of redox-labelled nucleotide(s), a vial of nucleotidyl transferase enzyme(s), an appropriate unlabelled nucleotide mix, an optimised reaction buffer, control template and primer so that the user may determine the efficiency of DNA synthesis. In this case, the user would supply specific primer and template nucleic acids for the application.

Electrochemical detection

Electrochemical detection can be employed in liquid chromatography, capillary electrophoresis and microchannel formats (see Kissinger and Heineman, Laboratory techniques in Electroanalytical Chemistry, Dekker, N.Y., 1996). It has been demonstrated that electrochemical detection is very sensitive, being able to measure amol to zmol quantities of sample in nl to pl volumes. Electrochemical methods have been used to detect labelled DNA during HPLC (Johnston, 1995; Shigenaga, 1990; Takenaka *et al.*, 1994), microcapillary electrophoresis (Woolley *et al.*, 1998) and in a microarray format (Umek *et al.*, 2001). In the Examples below HPLC-ECD has been used due to the local availability of instrumentation. The separation power of this method is low in comparison to CE, but is adequate for demonstration purposes.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not

the exclusion of any other element, integer or step, or group of elements, integers or steps.

Examples of the invention

The invention will now be described in connection with certain preferred embodiments in the following examples so that aspects thereof may be more fully understood and appreciated. It is understood that the examples are not intended to limit the invention to these particular embodiments.

Abbreviations:
DMF, dimethylformamide;
DMSO, dimethylsulfoxide;
DTT, dithiotreitol;
EDTA, ethylenediaminetetraacetic acid;
Fc-UTP, 5-(3-ferrocenecarboxamidopropenyl-1)-uridine 5'-triphosphate;
Fc-dUTP, 5-(3-ferrocenecarboxamidopropenyl-1)-2'-deoxyuridine 5'-triphosphate;
Fc-UMP, 5-(3-ferrocenecarboxamidopropenyl-1)-uridine 5'-monophosphate;
Fc-dUMP, 5-(3-ferrocenecarboxamidopropenyl-1)-2'-deoxyuridine 5'-monophosphate;
HBTU, O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate;
PAGE, polyacrylamide gel electrophoresis;
PCR, polymerase chain reaction;
RP HPLC, reverse phase high pressure liquid chromatography;
TEAB, triethylammonium bicarbonate;.

Materials and Methods for the Examples

Unless otherwise stated, starting materials for the chemical synthesis were obtained from Sigma-Aldrich or Bio-Rad and were used without further purification.

5-(3-aminopropenyl-1) uridine 5'-triphosphate and 5-(3-aminopropenyl-1) 2'-deoxyuridine 5'-triphosphate were prepared according to the reported procedure (Langer *et al.*, 1981).

Oligonucleotides were purchased from Sigma Genosis and purified by denaturing PAGE (20% acrylamide/8 M urea) as described (Sambrook *et al.*, 1989).

The Klenow fragment of *E. coli* DNA polymerase I was purchased from NEB. T4 DNA polymerase was from MBI Fermentas. T7 RNA polymerase was from USB. Tth DNA polymerase was from Perkin Elmer. ^{1}H and ^{31}P NMR spectra were recorded on Bruker DMX-300 spectrometer. Chemical shifts are reported in parts per million (δ) relative to an external standard.

UV spectra and DNA melting experiments were performed on a Cary 100 Bio spectrophotometer (Varian). HPLC separation and analyses were performed with an Akta Purifier system (Pharmacia Biotech) monitored at 260 and 440 nm. A reverse phase C18 column (Zorbax ODS, 250 - 9.4 mm) was utilized for preparative separations.

PAGE was run using a Protean II xi Cell (Bio-Rad) with 20 cm glass plates. Gels were run at 600 V in 0.09 M tris-borate, 2 mM EDTA running buffer and stained with SYBR Green II (Molecular Probes) before scanning with a Fluor-S MultiImager (Bio-Rad). Agarose gels were run at 5 V/cm in a Gello-tank cell (HyBaid) in 0.045 M tris-borate, 1 mM EDTA buffer.

HPLC analyses of ferrocene-labelled DNA samples with both optical and electrochemical detection were performed with a Shimadzu High Performance Liquid Chromatograph equipped with LC-10ATvp Solvent Delivery Module, SIL-10ADvp Auto Injector, DGU-14A degasser, SPD-M10Avp UV/VIS photodiode array detector, and ESA Coulochem II electrochemical detector (ESA, Inc.) (Guard Cell Model 5020 (potential – 0.8 V), Standard Analytical Cell Model 5010 (potential – 0.7 V)). A Vydac reverse phase column Protein & Peptide C18 (250 x 4 mm) was used for analyses.

Example 1: Synthesis of Fc-dUTP and Fc-UTP

A 45 μmol sample of 5-(*trans*-3-aminopropenyl-1) 2'-deoxyuridine 5'-triphosphate was evaporated twice from absolute ethanol to remove traces of water before dissolving in 1 ml anhydrous DMF. A solution of 23 mg (0.1 mmol) ferrocenecarboxylic acid in DMSO and 37.9 mg (0.1 mmol) solid HBTU were added to the nucleotide solution with stirring until dissolution of HBTU and the mixture incubated at room temperature overnight. The reaction mixture was diluted with 20 ml of 5 mM 2-mercaptoethanol in water and the yellow ferrocenecarboxylic acid precipitate removed with a 0.45 μm

polypropylene membrane filter (Gelman Sciences). The filtrate was applied to a DEAE-cellulose column (1 x 25 cm) equilibrated with 5 mM aqueous 2-mercaptoethanol and separated with a linear gradient of TEAB (0 – 0.35 M, 500 ml) in 5 mM 2-mercaptoethanol. Product eluted as a large peak at the end of the gradient.

The product fractions were pooled, evaporated, and purified by RP HPLC with a linear gradient of acetonitrile (0-30%) in 0.05 M $LiClO_4$. Solvent was removed by rotary evaporation, the residue dissolved in 0.5 ml water and the product precipitated by addition of 5 ml 2% $LiClO_4$ in acetone. The precipitate was washed with acetone and dried on air. Fc-dUTP yield 14 μmol (30%). UV (H_2O) λ_{max} = 439 nm (ε= 300 $M^{-1}cm^{-1}$). ^1H NMR (D_2O) δ 2.36 (m, H2', 2H), 3.98 (d, J = 4.5 Hz, H9, 2H), 4.19 (m, H4', H5', 3H), 4.27 (s, C_5H_5 of Fc, 5H), 4.51 (s, H2", 2H), 4.77 (m, H3', 1H), 4.81 (s, H1", 2H), 6.27 (t, J = 6 Hz, H1', 1H), 6.39 (s, H7, 1H), 6.48 (t, J = 4.5 Hz, H8, 1H), 7.88 (s, H6, 1H).

An identical procedure was used for the synthesis of Fc-UTP (yield 7 %).

Example 2: Characterisation

Ferrocene-labelled dUTP (Fc-dUTP, 1) and UTP (Fc-UTP, 2) derivatives (Fig. 1) were successfully synthesized by reaction of the 5-(3-aminopropenyl)-nucleoside triphosphates with ferrocenecarboxylic acid in the presence of HBTU. This procedure generates a relatively rigid 6-bond linkage between the nucleobase and redox label. The products were purified to homogeneity by ion-exchange chromatography followed by RP HPLC. The yields of both products were relatively low (30% for Fc-dUTP and 7% for Fc-UTP), probably due to steric hindrance in the course of the reaction. We have also used this procedure to synthesize a dUTP derivative adducted to ferroceneacetic acid.

Fc-dUTP and Fc-UTP have characteristic absorption spectra which correspond to a superposition of spectra for the modified nucleotide and ferrocene carboxamide constituents. They have strong absorption in the UV region and a weak, broad peak characteristic of ferrocene near 440 nm. Cyclic voltammetry of Fc-dUTP yields a symmetric peak with $E_{1/2}$ = 398 mV *vs.* Ag/AgCl, consistent with reversible redox reaction of the Fc moiety. The redox potential of Fc-dUTP is 90 mV greater than the potential of ferrocenecarboxylate (310 mV vs. Ag/AgCl) measured in the same buffer (data not shown), reflecting the change of pentadienyl ring substituent (-COO^- to -

CONHR) to one which is more electron-withdrawing. The observed potential is close to that reported for a ferrocene carboxamide moiety attached to the 5'-end of DNA oligonucleotides in aqueous buffer (406–425 mV vs. Ag/AgCl).

Example 3: Cyclic Voltammetry

Cyclic voltammograms were recorded with an electrochemical analyser (BAS). The three-electrode system consisted of a glassy carbon working electrode, a Ag/AgCl (saturated KCl) reference electrode (E_{ref} = 206 mV) and a platinum counter electrode. Experiments were performed in a 5 ml electrochemical cell containing 0.8 mM Fc-NTP in 20 mM tris-acetate (pH 7.4), 100 mM KCl, and 1 mM $MgCl_2$ at a scan rate of 20 mV/s. The scan range was from –0.1 to +0.8 V (*vs.* Ag/AgCl). (See Fig.2)

Example 4: Primer Extension by DNA Polymerases

A DNA partial duplex consisting of an 18-mer primer 5'-CAACGTCCGAGCAGTACA and a 40-mer template 5'-AAGCTCCTTAGTCTGTCAATGTACTGCTCGGACGTTGCGA (Fig. 3A) was prepared by annealing PAGE-purified oligonucleotides. DNA duplex (2 μM) was incubated in 20 μl polymerase reaction mixture (6.7 mM tris-HCl pH 8.8, 6.6 mM $MgCl_2$, 1 mM DTT, 16.8 mM $(NH_4)_2SO_4$, 200 μM dNTPs and 0.25 U/μl Klenow fragment or T4 DNA polymerase) for 20 min at room temperature. Reactions were stopped with an equal volume of gel loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanole FF), heated at 95 °C for 2 min and subjected to denaturing PAGE (see Fig. 3B).

The substrate properties of Fc-dUTP were tested in DNA polymerase-catalysed primer extension assays using the model DNA duplex shown in Fig. 3A. The sequence of the template allows the progress of primer extension to be controlled by omitting some dNTPs from the reaction mixture. The results of incubating primer-template with *E. coli* DNA polymerase I Klenow fragment or T4 DNA polymerase are shown in Fig 3B. Addition of unlabelled dTTP to the reaction mix results in extension of the 18-mer primer (lane 1) by 2 nucleotides (lanes 2 and 8). The product heterogeneity displayed by T4 DNA polymerase (lane 8) is caused by its stronger 3'-5' exonuclease activity, which is also evident in lanes 9-11. When Fc-dUTP replaces dTTP, both DNA polymerases incorporate two consecutive Fc-dUMP residues into the 3'-

end of the primer (lanes 3 and 9). Because the incorporated pFc-dU residue has a molecular weight almost twice that of the pT residue (574 vs. 321 Da) and the bulky adduct also alters the hydrodynamic properties of the chain, the mobility of the Fc-dUTP-extended primer is significantly lower than that of its natural counterpart. There is an indication that a small fraction of the primer is not extended by Klenow fragment (lane 3), but this behaviour is not consistent across the gel.

Some modified nucleoside triphosphates have the properties of terminators, their incorporation into DNA preventing or slowing further extension. To check this possibility, we incubated the primer-template with Fc-dUTP and one or two other dNTPs required for limited primer extension. In the presence of Fc-dUTP, dGTP and dATP, Klenow fragment successfully extends the chain following Fc-dU incorporation (lane 5). Similarly, T4 DNA polymerase extends the primer by three residues in the presence of Fc-dUTP and dGTP (lane 11). This allows us to conclude that Fc-dUTP is both efficiently incorporated and does not significantly inhibit further extension. Of some interest, Klenow fragment displays cleaner extension behaviour with the Fc-dUTP/dATP/dGTP mixture (lane 5) than with dTTP/dATP/dGTP (lane 4), where misincorporation at G15 has allowed the formation of a minor 26-mer product which terminates at the next "stop" position, G11. Incubation of the primer-template with all four natural dNTPs (lanes 6 and 12) or Fc-dUTP plus three dNTPs (lanes 7 and 13) allows run-off extension of the primer. Again no visible termination sites were registered when Fc-dUTP replaced dTTP.

Example 5: DNA Labelling with Fc-dUTP in the Course of PCR

A segment of the T4 DNA ligase gene (positions 1001 to 1988) was used as a model sequence for amplification in the presence of a ferrocene-labelled TTP analogue. The gene was cloned into plasmid pKL01. The 25-mer 5'-GCT GAT GGA GCT CGG TGT TTT GCT T-3' was used as a forward primer, and 31-mer 5'- TAT ATA AGC TTC ATA GAC CAG TTA CCT CAT G –3' was used as a reverse primer. The use of these primers allows formation of a 998 nt long amplicon. The reaction mixtures (20 uL each) contained 6.7 mM tris-HCl (pH 8.8), 1.66 mM $(NH_4)_2SO_4$, 0.045% Triton X-100, 0.02 mg/mL gelatin, 2.5 mM $MgCl_2$, 0.2 uM each primer, 20 ug/mL pKL01 plasmid, 0.2 mM dNTPs, and 0.1 U/uL *Tth* polymerase (exo$^-$). In some reaction mixtures, TTP was

partially or fully substituted with Fc-dUTP in such a way that the total concentration of TTP and Fc-dUTP was still 0.2 mM. Conditions of PCR were as follows: 2 min at 94 °C, and then 22 cycles at 94 °C for 30 sec, 50 °C for 1 min, and 70 °C for 10 min. After amplification, 4 uL of gel loading buffer (30% glycerol, 0.25% bromphenol blue and 0.25% xylene cyanole FF)was added, and samples were analysed on 1% agarose gel.

Full substitution of TTP by Fc-dUTP did not support the formation of a PCR product by *TTh* DNA polymerase. However, when TTP was substituted by Fc-dUTP at 25%, 50%, or 75%, synthesis of the correct amplicon was observed. The amplicon molecular showed a proressive increase in molecular weight with increasing Fc-dUTP:dTTP ratio, indicating extensive Fc-dUMP incorporation. .

Example 6: Incorporation of Fc-UTP into RNA in the course of transcription.

Circular plasmid pT7Mta which contains the promoter for T7 RNA polymerase followed by the gene for aptamer C40 and a T7 terminator sequence was used for transcription. T7 RNA polymerase tends to produce short abortive RNA transcripts when modified nucleotides are incorporated into the first 12 nucleotides of RNA. To avoid this potential complication, we used a template which doesn't contain any A residues in the first 18 nucleotides of the coding sequence.

A typical transcription mixture (10 μL) contained 40 mM tris-HCl (pH 8.0), 15 mM $MgCl_2$, 5 mM DTT, 0.05 mg/mL BSA, 1 U/□L Rnasin, 0.4 mM NTPs, 10 ug/mL pT7Mta template, and 10 U/uL T7 RNA polymerase. In some reaction mixtures, UTP was partially or fully substituted with Fc-UTP in such a way that the total concentration of UTP and Fc-UTP was still 0.4 mM. Reaction mixtures were incubated at 37 °C for 2 h. Reactions were stopped by addition of 10 μL of gel loading buffer (98% formamide, 10 mM EDTA (pH 8.0), 0.025% bromphenol blue and 0.025% xylene cyanole FF) and heated at 95 °C for 2 min. RNA fragments were separated by 10% PAGE/8 M urea. The gel was stained by SYBR Green II (Sigma) according to manufacturer's procedure, and visualized on a Fluorimager (Bio-Rad).

Due to inefficient T7 termination in this construct, a majority of the RNA products formed are significantly longer than the intended 117 nt long product. Nonetheless, large quantities of Fc-UMP-labelled RNA were produced. Substitution of UTP by Fc-UTP caused a significant decrease in

the amount of RNA product formed. However, even in the total absence of UTP, T7 RNA polymerase synthesized a significant amount of RNA product.

Example 7: Electrochemical detection of labelled polynucleotides during HPLC.

4 μM duplex DNA (40-mer template 5'-AAGCTCCTTAGTCTGTCAATGTACTGCT CGGACGTTGCGA-3' and 18-mer primer 5'-CAACGTCCGAGCAGTACA-3') was incubated in 240 μL of reaction mixture consisting of 6.7 mM tris-HCl (pH 8.8), 6.6 mM $MgCl_2$, 1 mM DTT, 16.8 mM $(NH_4)_2SO_4$, 200 μM dNTPs (except TTP), 200 μM Fc-dUTP, and 0.25 U/μL of Klenow fragment for 20 min at room temperature. Low molecular weight components were separated on Bio-Spin 30 chromatography column (Bio-Rad). The eluate was extracted with equal volumes of phenol/chloroform (1:1) and chlorophorm. DNA was precipitated by addition of 10 volumes of 2% $LiClO_4$ in acetone and centrifugation (12000g, 15 min). Precipitate was dried *in vacuo*, redissolved in 200 μL of HPLC buffer (50 mM $LiClO_4$ / 2.5% acetonitrile in water), and the DNA concentration was determined spectrophotometrically by absorption at 260 nm. Different amounts of sample were loaded onto the analytical reverse-phase column (Vydac, Protein & Peptide C18, 250 × 4 mm) and analysed by isocratic elution with optical (260 nm) and electrochemical (E = 0.7 V) detections (flow rate – 1 mL/min). After being extended in the presence of all 4 dNTPs including Fc-dUTP instead of TTP, the model DNA duplex would contain five Fc-dUMP residues. We used this ferrocene-labelled duplex for electrochemical detection in the course of RP HPLC. The HPLC system was equipped with both optical and electrochemical detectors as described in Materials and Methods. Different quantities of DNA duplex were injected on the reverse phase column and eluted in isocratic mode by 50 mM $LiClO_4$ / 2.5% acetonitrile in water. The eluate was monitored optically at 260 nm and electrochemically at 0.7 V. In our conditions, the retention time for DNA duplex was 17.5 min. Only picomolar quantities of DNA were reliably detected with UV/VIS photo array detector, while electrochemical detection allowed to register femtomolar amounts of the duplex (Fig. 4).

Example 7: Melting analysis of DNA duplexes containing Fc-dUMP residues

DNA samples for melting experiments were prepared as described above in the preparation of DNA duplex for electrochemical detection. As a

control sample, unmodified DNA duplex containing all natural nucleotides was prepared using the same procedure. Both DNA duplexes were dissolved in 1 mL of 0.3 M KH_2PO_4 (pH 7.0) and transferred into standard quartz cuvettes. The melting curves were obtained by recording the changes in absorption of samples at 260 nm with increase of temperature from 25 °C to 95 °C (temperature gradient 1°C per min)

Modification of natural components of nucleic acid can sometimes severely affect the stability of the DNA duplex. This issue is very important for all applications where formation of DNA hybrids is involved. To check the effect of incorporation of ferrocene-modified nucleotides into DNA, we have measured the melting temperature of a DNA duplex containing 5 residues of Fc-dUMP. The melting of unmodified duplex with the same sequence was studied for comparison. The melting temperature of modified DNA hybrid (71 °C) is only 4 degrees lower then the one of normal duplex (75 °C). This allows us to conclude that modification by ferrocene at the C5 position of dUMP does not significantly disrupt the native structure of DNA.

Industrial application

Conjugation of ferrocene and other redox-active moieties with nucleoside triphosphates enables the broad expansion and diffusion of electrochemical methodologies in molecular biology and genetic analysis. Enzymatic redox labelling of nucleic acids has a range of applications in DNA sequencing, mRNA expression analysis and genotyping.

References

Burgess, K. and Cook, D. (2000) Synthesis of nucleoside triphosphates. *Chem. Rev.* 100: 2047-2059.

Langer, P. R., Waldrop, A. A. and Ward, D. C. (1981) Enzymatic synthesis of biotin-labelled polynucleotides: novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA* 78: 6633-6637.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY., Cold Spring Harbor Laboratory Press.

Johnston, D. H. (1995) Electrochemical measurement of the solvent accessibility of nucleobases using electron transfer between DNA and metal complexes. *J. Am. Chem. Soc.* 117: 8933-8938.

Shigenaga, M. K. (1990) In vivo oxidative DNA damage: measurement of 8-hydroxy-2'-deoxyguanosine in DNA and urine by high-performance liquid chromatography with electrochemical detection. *Methods Enzymol.* 186: 521-530.

Takenaka, S., Uto, Y., Kondo, H., Ihara, T. and Takagi, M. (1994) Electrochemically active DNA probes: detection of target DNA sequences at femtomole level by high-performance liquid chromatography with electrochemical detection. *Anal. Biochem.* 218: 436-443.

Umek, R. M., Lin, S. W., Vielmetter, J., Terbrueggen, R. H., Irvine, B., Yu, C. J., Kayyem, J. F., Yowanto, H., Blackburn, G. F., Farkas, D. H. and Chen, Y.-P. (2001) Electronic detection of nucleic acids. A versatile platform for molecular diagnostics. *J. Mol. Diag.* 3(2): 74-84.

Woolley, A. T., Lao, K. Q., Glazer, A. N. and Mathies, R. A. (1998) Capillary electrophoresis chips with integrated electrochemical detection. *Anal. Chem.* 70(4): 684-688.

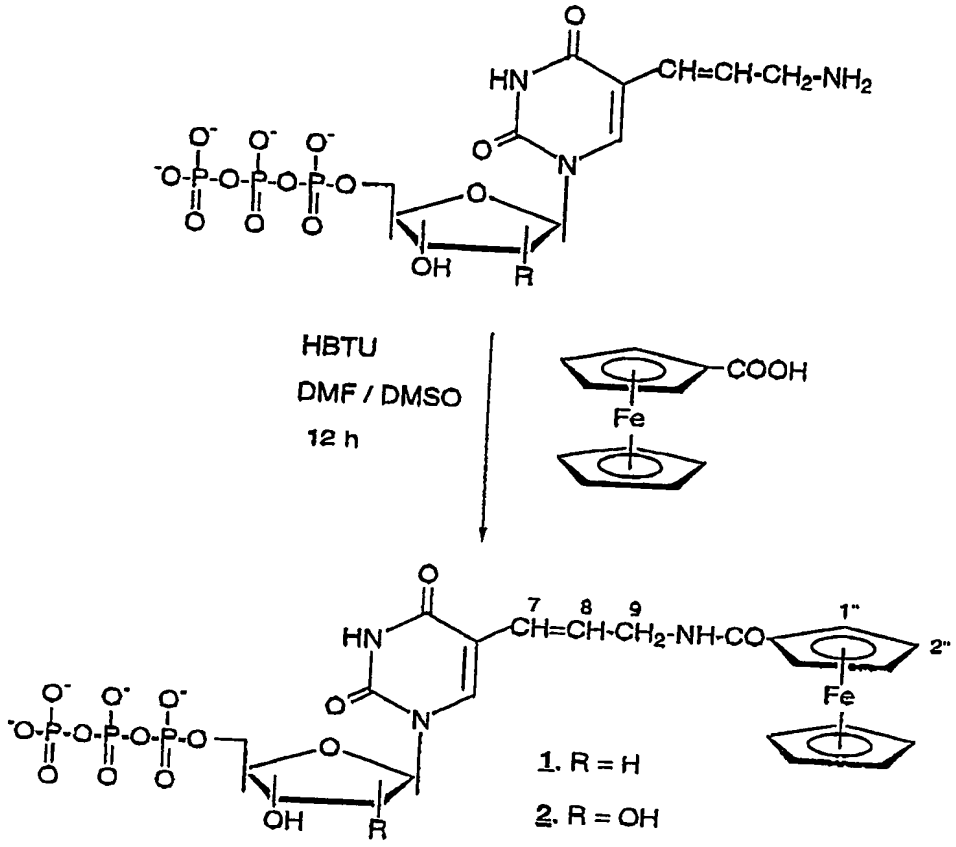

HBTU
DMF / DMSO
12 h
COOH
Fe
HN
CH=CH-CH2-NH2
CH=CH-CH2-NH-CO
1. R = H
2. R = OH

Fig 1

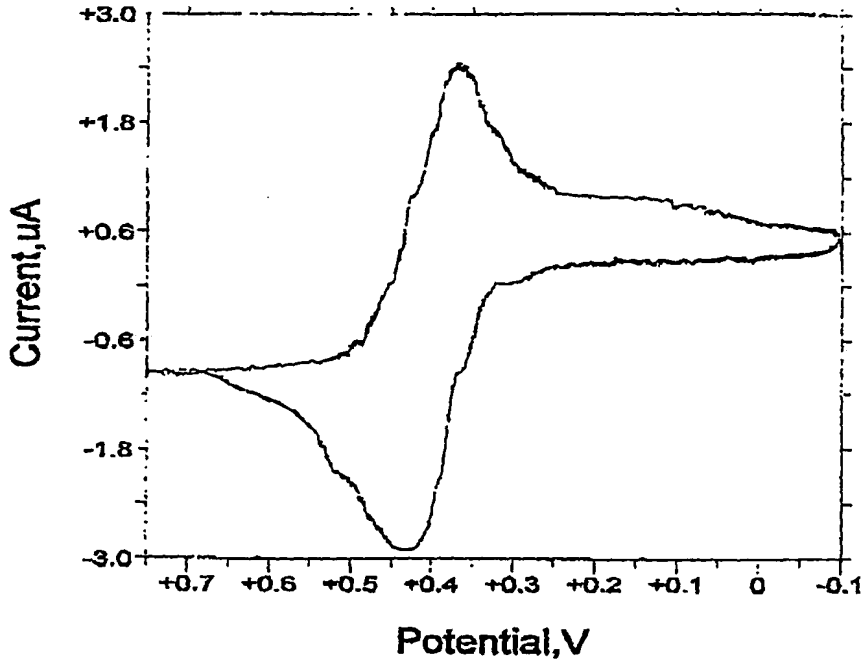


Fig 2

```
              40     35     30     25     20    15    10     5   1
3'-AGCGTTGCAGGCTCGTCATGTAACTGTCTGATTCCTCGAA-5'
      5'-CAACGTCCGAGCAGTACA
```

Extension by 2 nt in the presence of TTP or Fc-dUTP *t t*

Extension by 3 nt in the presence of TTP (Fc-dUTP) and dGTP *t t g*

Extension by 4 nt in the presence of TTP (Fc-dUTP), dGTP and dATP *t t g a*

Full copying of template in the presence of all four dNTPs *t t g a c a g a c t a a g g a g c t t*

Fig 3A

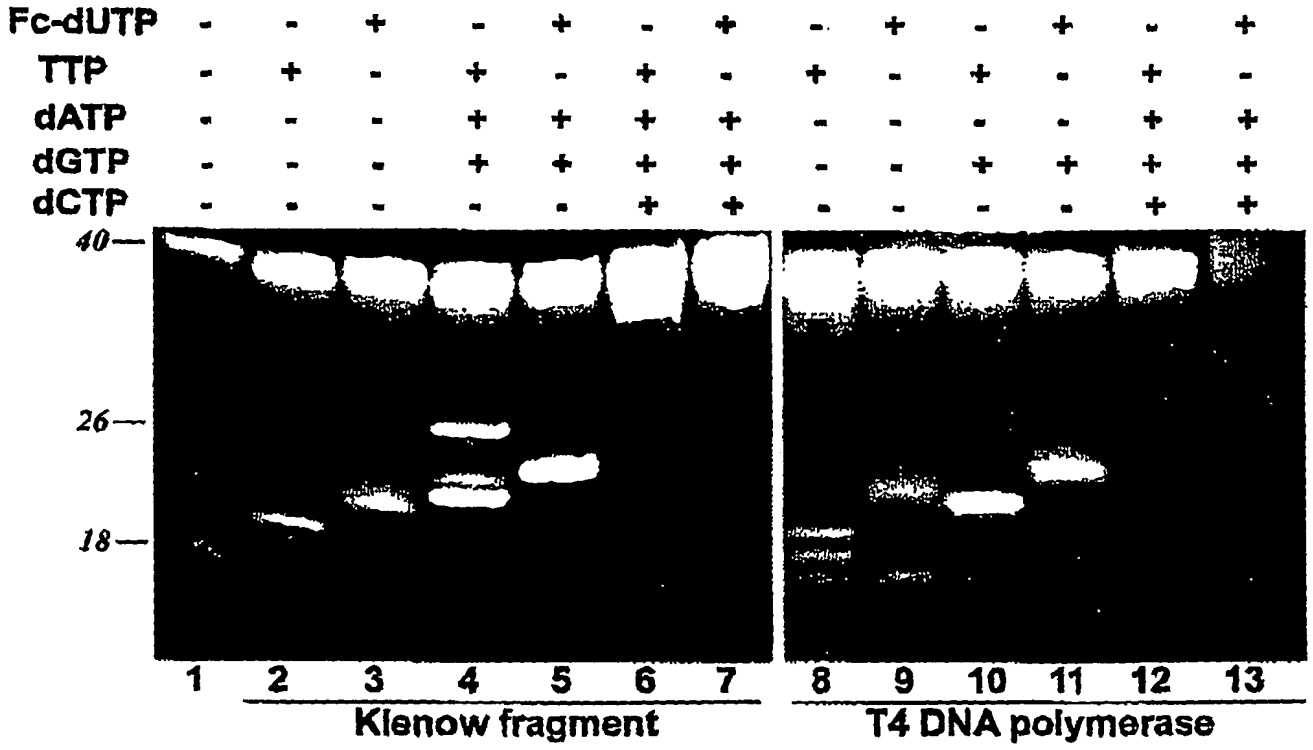
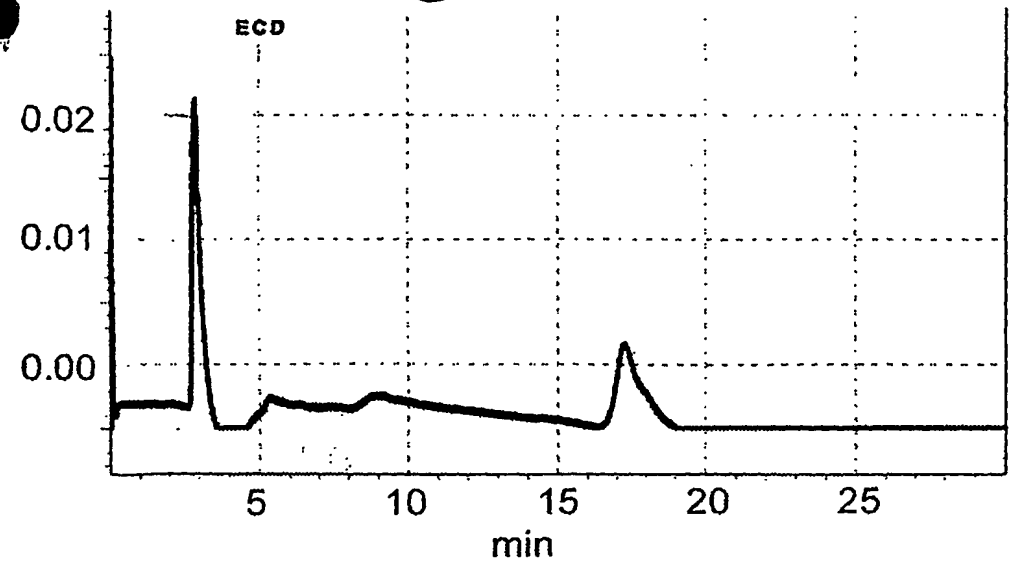

Fc-dUTP - - + - + - + - + - + - +
TTP - + - + - + - + - + - + -
dATP - - - + + + + - - - - + +
dGTP - - - + + + + - - + + + +
dCTP - - - - - + + - - - - + +
40
26
18
1 2 3 4 5 6 7
Klenow fragment
8 9 10 11 12 13
T4 DNA polymerase

Fig 3B


ECD
0.02
0.01
0.00
V
5
10
15
20
25
min

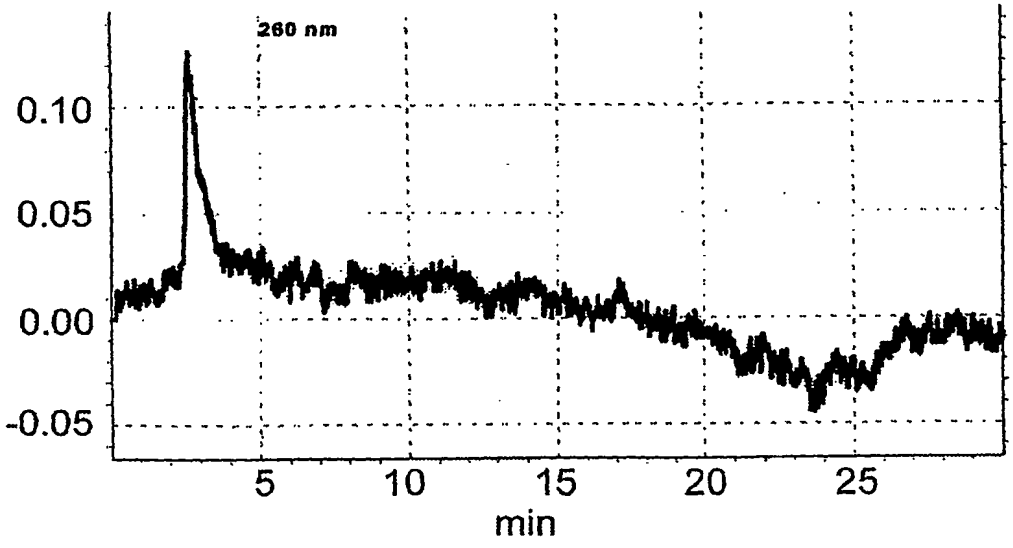

260 nm
0.10
0.05
0.00
-0.05
mAU
5
10
15
20
25
min

Fig 5